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Anti-synthetic peptide antibody reacting at the fusion junction of deletion–mutant epidermal growth factor receptors in human glioblastoma

(protooncogene/*c-erbB*/peptide immunization/brain tumor)

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ABSTRACT **We have investigated human gliomas that amplify and rearrange the epidermal growth factor receptor gene, with generation of an in-frame deletion mutation of 802 nucleotides in the external domain. This in-frame deletion mutation generates a local amino acid sequence at the fusion junction of what normally were distant polypeptide sequences in the intact epidermal growth factor receptor. This 14-amino acid peptide was chemically synthesized, coupled to keyhole limpet hemocyanin, and used as an immunogen in rabbits. The elicited antibody reacted specifically with the fusion peptide in ELISA. The anti-fusion junction peptide antibody was purified by passage of the antiserum over a peptide affinity column with acidic elution. The purified antibody selectively bound the glioma deletion mutant as compared to the intact epidermal growth factor receptor as assessed by immunocytochemistry, immunofluorescence, immunoprecipitation with gel electrophoresis, and binding experiments using radioiodinated antibody. These data indicate that it is feasible to generate site-specific anti-peptide antibodies that are highly selective for mutant proteins in human tumors. The anti-peptide antibody described here, and other mutation site-specific antibodies, should be ideal candidates for tumor immunoimaging and immunotherapy.**

Protooncogenes are normal eukaryotic cellular genes that may be activated into transforming genes by mutational events, including point mutations and deletion mutations. These mutations are attractive targets for cancer therapy, as they should be found only in tumor tissues and not in normal tissues. One approach to exploit these tumor-specific structural alterations is to generate site-specific anti-peptide antibodies directed against the mutation site. Such antibodies have been generated against the activated *ras* p21 protooncogene where an anti-point mutant peptide antibody reacted specifically with *ras* p21 protein containing an arginine at position 12 but did not react with the normal glycine-12 protein (1). The antibody detected the mutant arginine-12 p21 oncoprotein in breast tumors of v-H-*ras* transgenic mice but not in normal tissues. A second reported anti-point mutant peptide antibody recognized the oncogenic *ras* p21 protein with a serine at position 12 but did not bind the mutant arginine-12 p21 or the normal glycine-12 p21 (2). The mutant *ras* protein is, however, not a cell-surface protein and as such may be less accessible to therapeutic targeting strategies such as immunotherapy. For immunotherapeutics, a cell-surface mutant protein would be more accessible and should represent a superior structural target.

Here we report an anti-peptide antibody that reacts with the fusion junction of a second type of structural mutation, the deletion mutation in the epidermal growth factor receptor (EGFR), which occurs in the most common and most malignant of the human primary brain tumors, the glioblastoma multiforme. The EGFR gene is the protooncogene of v-*erbB* and is often amplified and overexpressed in malignant human gliomas (3–6). Often associated with this amplification is structural rearrangement of the gene (3–6), which results in the expression of truncated EGFR proteins (6–8). Recently, these rearrangements have been shown to result in deletions in the EGFR gene, thus accounting for the expression of the truncated proteins (9, 10). The deletions in the glioma EGFR gene so far detected segregate into distinct classes based on the size and location of the deletion. One class of deletion mutation that has been detected in three glioma patients by molecular biologic techniques (9) resulted in an 802-base-pair in-frame deletion with generation of a glycine residue at the fusion point (see Fig. 1). A 14-amino acid peptide corresponding to the amino acid sequence at the fusion junction generated by this deletion was chemically synthesized. We immunized rabbits with peptide–keyhole limpet hemocyanin (KLH) conjugate, purified the antibody from the rabbit antiserum on a peptide affinity column, and characterized the reactivity of the antibody. This site-specific anti-mutant EGFR peptide antibody is highly selective for the deletion mutant EGFR protein compared to the intact EGFR protein. The selective targeting of cell-surface mutant proteins in human tumors, as exemplified by the anti-mutant EGFR antibody, represents an unusual and potentially important approach to the diagnosis and treatment of human cancer.

MATERIALS AND METHODS

Peptides and Immunizations. A 14-amino acid peptide corresponding to the predicted amino acid sequence at the fusion junction based on nucleotide sequence was chemically synthesized and purified, and a portion was coupled to KLH by Applied Biosystems. Purity of the peptide was confirmed by HPLC; structural characterization was performed by amino acid analysis and mass spectroscopy (at Applied Biosystems). The peptide, shown in Fig. 1, represents a unique sequence generated by fusion of normally distant polypeptide

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Abbreviations: EGFR, epidermal growth factor receptor; KLH, keyhole limpet hemocyanin.
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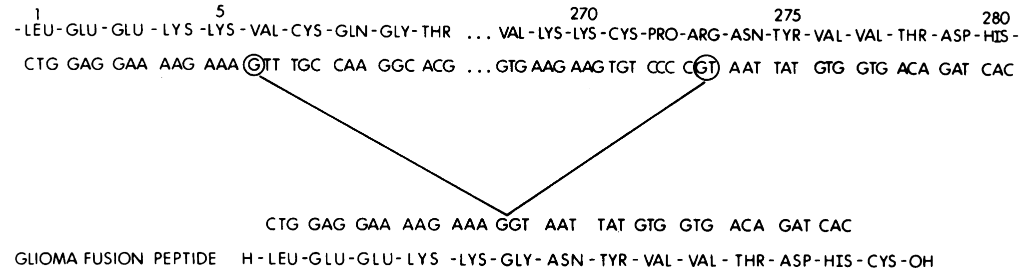

FIG. 1. Amino acid sequence of glioma fusion junction peptide. An 802-base-pair in-frame EGFR gene deletion (*Upper*) results in the fusion of normally distant EGFR gene and protein sequences (*Lower*). A glycine residue is created at the fusion point. The 13-amino acid fusion junction sequence was chemically synthesized (Applied Biosystems) with a carboxyl-terminal cysteine for conjugation to KLH, a carrier molecule for immunizations.

sequences; a search of GenBank did not identify this sequence in any known human gene.

Three New Zealand White rabbits were immunized with peptide conjugated to KLH. On day 0, subcutaneous immunization was administered with 250 μg of peptide conjugated to KLH in 1 ml of a 1:1 emulsification of phosphate-buffered saline (PBS) and complete Freund's adjuvant. Each rabbit was injected with this dose at four separate sites. On day 33, each rabbit was given a booster injection at four separate sites with 50 μg of peptide conjugated to KLH in 1 ml of a 1:1 emulsification of PBS and incomplete Freund's adjuvant. Antisera were obtained by bleeding the animals on days 40 and 43.

Antibody titers against peptide were determined by ELISA essentially as described (11).

Purification and Characterization of Anti-Peptide Antibody. The anti-peptide antibody was purified from antiserum by a peptide-Sepharose affinity column with elution by acidic pH. The affinity column was prepared by coupling 5 mg of peptide to cyanogen bromide-activated Sepharose (Sigma), as described in the Pharmacia protocol. The extent of coupling was 100%, as determined by BCA protein assay (Pierce) of the solution overlying the gel. Ten milliliters of antiserum from rabbit 396 was passed over the column, and the column was washed extensively with 500 ml of PBS. Elution was with 100 mM glycine buffer (pH 2.5) with immediate neutralization into 0.4 M Hepes buffer (pH 7.4). Control IgG was purified from rabbit preimmune serum by protein A-Sepharose affinity chromatography. The size and homogeneity of the purified antibodies were monitored under nondenaturing conditions by size exclusion HPLC and under denaturing conditions by SDS/PAGE. HPLC was performed on a calibrated Waters 300 SW column (1 × 30 cm) (previously calibrated with standards in the size range of 20–400 kDa). Protein elution was followed at 215 nm. SDS/PAGE utilized a 10% resolving gel in the SDS discontinuous buffer system of Laemmli (12). The protein bands were visualized with Coomassie blue staining.

The affinity-purified antibody was characterized in immunocytochemistry by using frozen tissue sections and the avidin-biotin complex method as described (6). The tissues tested included a range of normal fetal and adult tissues, carcinomas (prostatic, bladder, breast, and lung), glioma biopsies (-Bx), and gliomas grown in xenograft form in nude mice (-X). The normal tissues, carcinomas, and glioma biopsies were from the Duke University Medical Center Tissue Bank. The glioma xenografts were grown as described (6). The affinity-purified anti-peptide antibody and the purified preimmune rabbit control IgG were used at a concentration of 1–2 μg/ml, as determined by initial titration experiments. The antibody could be used in antiserum form with an optimal titer in immunocytochemistry of 1:3000. $F(ab')_2$ fragments of normal rabbit IgG and the anti-peptide antibody were also tested in immunocytochemistry on glioma D-270 MG-X and skin. $F(ab')_2$ fragments were generated and purified as described (13). Also used in immunocytochemistry of the glioma biopsies was rabbit anti-peptide antibody reactive with the intact EGFR (product no. 0A-11-852; Cambridge Research Biochemicals, Valley Stream, NY). This antiserum was used at a dilution of 1:1000, as established by initial titration experiments.

The purified anti-peptide antibody and preimmune IgG were both labeled with ^{125}I at a specific activity of ≈1.6 μCi/μg (1 Ci = 37 GBq) using a variation of the Iodo-Gen method as described (13). Radioiodinated proteins (22 ng; 50,000–70,000 cpm) were incubated for 2 hr at 4°C in triplicate with 200 μl of 20 mM Hepes (pH 7.4) containing 0.1% bovine serum albumin and 40 μl of a 1-mg/ml suspension of microsomal membranes. The membranes tested were as follows: D-270 MG-X, a glioma tumor expressing the in-frame deletion mutant EGFR; A431-X, a squamous cell carcinoma overexpressing the intact EGFR; D-245 MG-X, a tumor containing or expressing an EGFR molecule that lacks most of the extracellular domain and serves as a negative tissue control. After the incubation period, the membranes were separated from unbound activity by using 0.22-μm cellulose acetate centrifuge filter units (Spin-x; Costar) washed twice with 1 ml of the incubation buffer. The filters had been pretreated by a 30-min incubation at room temperature followed by three washes with buffer. Using this procedure, nonspecific binding of radioactivity to the filters was <0.2%. Filters and washes were assayed for ^{125}I activity in similar counting geometries with an automated γ-counter.

The molecular specificity of the anti-peptide IgG was tested by immunoprecipitation reaction. The binding in this reaction was followed by EGFR autophosphorylation, SDS/PAGE, and autoradiography essentially as described (6). Monoclonal antibody 528 (Ab-1; Oncogene Science, Manhasset, NY) was used as a positive control, as it will immunoprecipitate both intact and mutant EGFR (6).

Binding of anti-peptide IgG to live tumor cells was determined by immunofluorescence assay. D-270 MG-X or A431-X cells were prepared by dissociation from tumor xenografts grown subcutaneously in athymic mice. After collagenase treatment and washing, 10% normal goat serum was used to block nonspecific binding. The cells were incubated 30 min at 4°C with 100 μl of anti-peptide (20 μg/ml) or preimmune IgG. After incubation with fluorescein-labeled goat anti-rabbit IgG, the immunofluorescence was visualized with a Zeiss microscope.

The effect of purified antibody on EGFR kinase was determined by phosphorylation of EGFR in D-270 MG-X and A431-X membranes as described by Davis and Czech (14).

RESULTS

Production of Anti-Peptide Antisera. All three rabbits immunized with peptide–KLH conjugate exhibited a marked

IgG response as assessed by ELISA against uncoupled peptide bound to plates (Fig. 2). The half-maximal titers (the antiserum dilution at one-half maximal absorbance) varied from 1:6000 to 1:50,000. The binding reaction was specific, as the preimmune serum from each rabbit was nonreactive; a nonreactive baseline was also obtained when the antisera reacted with a second 14-amino acid peptide of different sequence (Pep-1: H-Asn-Leu-Leu-Glu-Gly-Cys-Thr-Gly-Pro-Gly-Leu-Glu-Gly-Cys-OH). The half-maximal titer of purified antibody in this assay was 0.5 μg of anti-peptide IgG per ml of PBS.

Purification of Anti-Peptide IgG. Elution of anti-peptide antibody from a peptide affinity column was accomplished by using glycine buffer (pH 2.5). A yield of 6 mg of pure IgG was obtained from a starting load of 10 ml of rabbit antiserum 396 (the antiserum with the 1:50,000 half-maximal titer). Lower yields were obtained with purification of anti-peptide IgG from antisera of lower half-maximal titers. Elution of active antibody could also be accomplished with 3.5 M $MgCl_2$ (pH 3.5 or 6.5). The activity of the antibody eluted under the different conditions was similar, as assessed by ELISA against free peptide. The eluted antibody was identified as IgG by apparent molecular weights on a size exclusion HPLC column and SDS gel (data not shown). Purity was >98%.

Reaction of Anti-Peptide IgG with Mutant EGFR but Not Intact EGFR. The anti-peptide antibody reacted with mutant EGFR in a detergent-solubilized state, as judged by immunoprecipitation, with autophosphorylation and SDS/PAGE (Fig. 3). The positive control immunoprecipitation was with monoclonal antibody 528, which is directed against the EGFR external domain (15–18). This antibody reacted with both the intact A431-X and mutant D-270 MG-X EGFR, as reported (6). The anti-peptide antibody, in contrast, specifically immunoprecipitated the 145-kDa mutant EGFR in glioma D-270 MG-X and failed to immunoprecipitate the intact A431-X EGFR. Purified preimmune IgG did not immunoprecipitate the mutant EGFR.

Immunocytochemical Detection of Mutant EGFR Expression. The anti-peptide IgG also reacted with the mutant EGFR in frozen tissue sections fixed briefly with acetone. The antibody specifically recognized only the mutant glioma EGFR and not the intact A431-X squamous cell carcinoma EGFR in both xenograft (Fig. 4) and biopsy tissue. This immunostaining of the mutant EGFR was specific, as purified preimmune IgG was nonreactive, and preincubation of anti-peptide IgG with excess peptide blocked the immunocytochemical staining. In both glioma biopsy and xenograft tissues, which bound the anti-peptide antibody, the immunostaining was localized to the cytoplasm and cell surface; virtually every tumor cell exhibited immunoreactivity.

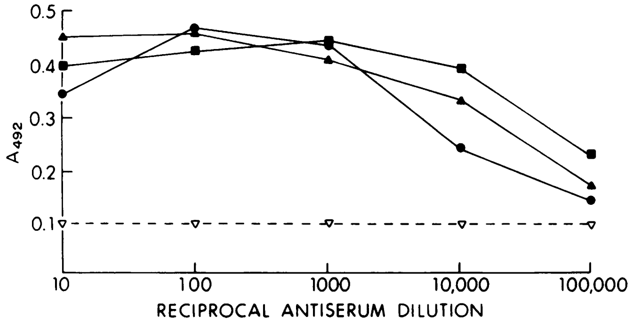

FIG. 2. Reactivity of antisera from three rabbits (●, ▲, and ■) with fusion junction peptide in ELISA. Antibody titers were determined against free peptide bound to polyvinyl chloride plates. Absorbance values represent the means of triplicate determinations. Nonspecific binding (▽) was determined by the use of preimmune serum and reaction of the anti-peptide antibody with a 14-amino acid peptide of irrelevant sequence.

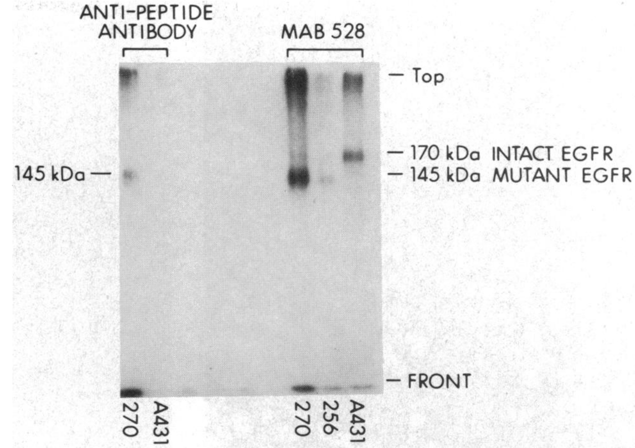

FIG. 3. Immunoprecipitation of mutant EGFR, but not intact EGFR, by anti-peptide antibody. Monoclonal antibody 528 (product Ab-1; Oncogene Science) was used to immunoprecipitate Triton X-100 detergent-solubilized intact A431-X EGFR and mutant D-256 MG-X and D-270 MG-X EGFRs, with ^{32}P autophosphorylation and SDS/PAGE with a 7.5% resolving gel (right three lanes). The gel was exposed to x-ray film at −70°C. Anti-peptide antibody immunoprecipitated the mutant glioma D-270 MG-X EGFR but not the intact A431-X EGFR (left two lanes).

Screening of normal and neoplastic tissues by immunocytochemistry with the anti-peptide antibody revealed positivity only in a subset of glioblastomas. Immunoreactive mutant EGFR was identified in two human glioma biopsies (D-270 MG-Bx and D-317 MG-Bx) and the corresponding xenografts (D-270 MG-X and D-317 MG-X) known to amplify the mutant EGFR gene and express mutant EGFR protein. Four additional glioblastoma biopsies exhibited immunostaining with the anti-peptide antibody; one of these (D-397 MG-Bx) was subsequently tested by an RNA-based polymerase chain reaction assay and was shown by sequencing to possess the same deletion mutation as gliomas D-270 MG-Bx and D-317 MG-Bx. The structure of the EGFR gene in the others is currently being analyzed.

Further evidence for the selectivity of this antibody was obtained in this screen of glioma biopsies, as 27 of 27 biopsies reacted specifically with rabbit anti-peptide antibody against the intact EGFR; but only those gliomas with the proven (three cases) or suspected (three cases) deletion mutation reacted with the anti-fusion junction peptide antibody. A range of fetal and adult normal tissues and carcinomas failed to react with the anti-peptide antibody, as assessed by this method.

Binding of ^{125}I-Labeled Anti-Peptide IgG to Tumor Membranes Expressing Intact and Mutant EGFR. Specific direct binding of radioiodinated anti-peptide IgG to membranes expressing the D-270 MG-X mutant EGFR was significantly higher at 28.2% than to membranes expressing intact EGFR (in A431-X) at 4.9% or a v-*erbB*-like EGFR (in D-245 MG-X) at 3.8%. The specificity of the anti-peptide binding reaction with demonstrated by assessing nonspecific binding with ^{125}I-labeled purified preimmune IgG; nonspecific binding ranged from 2% to 3.6%.

Effect of Anti-Peptide IgG on Mutant EGFR Kinase Activity. The anti-synthetic peptide antibody did not affect basal or EGF-stimulated intrinsic mutant EGFR kinase activity. The kinase activity of the mutant EGFR did not change with the presence of antibody, as assessed by band intensity in autoradiography of an SDS/polyacrylamide gel (data not shown).

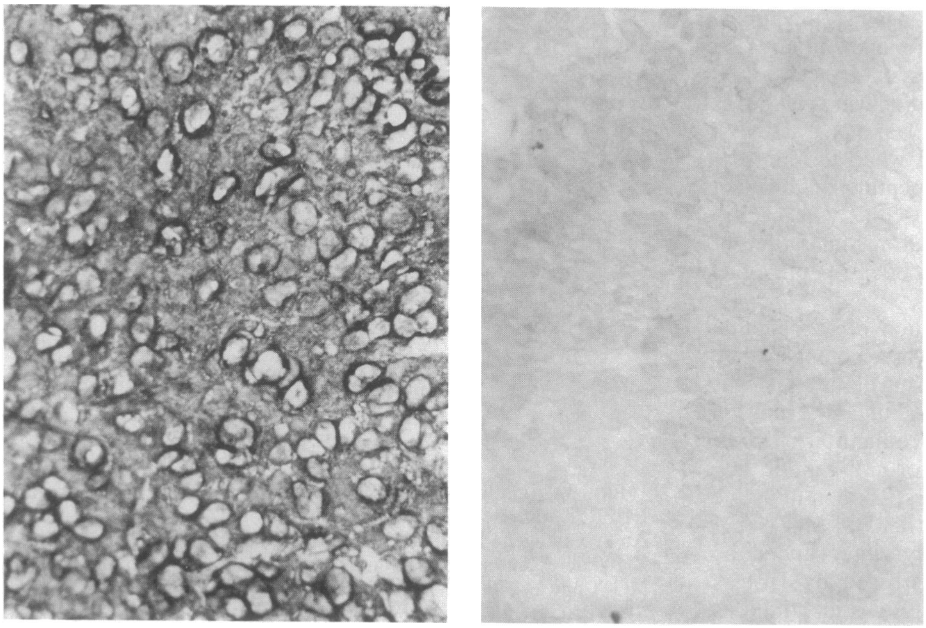

FIG. 4. Reaction of anti-peptide antibody with glioma D-270 MG-X expressing mutant EGFR (*Left*) and lack of binding with squamous cell carcinoma A431-X expressing intact EGFR (*Right*). The dark cytoplasmic immunostaining indicates a positive reaction. Immunocytochemistry was done on frozen sections of xenograft tumors, with diaminobenzidine as the chromagen in the avidin-biotin complex method. Anti-peptide antibody reacted with tissue sections at a concentration of 2 μg per ml of PBS. Hematoxylin counterstain. ($\times 280$.)

Internalization of Anti-Peptide IgG. The anti-synthetic peptide antibody rapidly bound the surface mutant EGFR expressed on live glioma D-270 MG-X cells, and this binding was reflected by a rimming pattern of the immunofluorescent secondary antibody label. Internalization rapidly ensued and was manifested as a speckled intracytoplasmic morphology. Internalization with loss of the peripheral plasma membrane rimming was complete by 60 min at 37°C.

DISCUSSION

The data presented here demonstrate the feasibility of generating a site-specific anti-synthetic peptide antibody that reacts at the fusion junction of an in-frame deletion mutant human oncoprotein. Previous work with other oncoproteins, most notably mutant *ras* p21 proteins (1, 2), has demonstrated that even a single amino acid change due to a point mutation is sufficient to generate an antibody that will react with the mutant but not the normal protein. In-frame internal deletion mutations also generate a different local peptide sequence, and we exploited sequence data obtained by sequencing subclones of polymerase chain reaction products of cDNA fragments from the mutated alleles (9) to design the fusion junction peptide for chemical synthesis. We selected a 13-amino acid peptide corresponding to normal EGFR peptides of 5 and 7 amino acids on the amino-terminal and carboxyl-terminal sides, respectively, of an amino acid (glycine) generated at the point of fusion. A cysteine residue was added at the carboxyl-terminal end for coupling to KLH with *N*-succinimidyl bromoacetate used as a cross-linker. The basis for this peptide design was that the 13-amino acid peptide should have been of a sufficient length to generate an antibody response, while it was hypothesized that the two linear stretches of 5 and 7 amino acid peptides of normal sequence may not have been of sufficient length to generate an immune response. In general, synthetic peptides with <10 amino acid residues fail to elicit an effective antibody response (19). The 5-amino acid sequence on the amino-terminal side of the fusion point amino acid glycine also represents the 5 amino-terminal residues in the normal EGFR (20), and thus the fusion junction synthetic peptide has the same sequence as the new amino terminus of the mutant EGFR. The specificity of the anti-peptide antibody in reacting selectively with the mutant 145-kDa EGFR compared to the intact 170-kDa EGFR may then be due to both the fusion of the normally distant 5- and 7-amino acid peptides and also to the generation of a glycine residue at the fusion point.

The selective reaction of the anti-peptide antibody with the mutant EGFR protein compared to the intact EGFR protein was established by several lines of experimental evidence. The anti-peptide antibody immunoprecipitated only the mutant EGFR and not the intact EGFR (Fig. 3); the antibody bound only those tissues expressing the mutant EGFR and not tissues known to express high levels of the intact EGFR, such as skin, liver, and A431-X squamous cell carcinoma (Fig. 4). Furthermore, radioiodinated anti-peptide antibody specifically bound to the mutant EGFR in membranes to a much higher degree than the negative control membranes and the intact EGFR in A431-X carcinoma membranes. The slight (4.9%) specific binding to A431-X squamous cell carcinoma membranes does not, however, entirely exclude minimal reactivity with the intact EGFR.

An anti-peptide antibody such as the one described here is potentially useful at both basic and clinical levels. First, this anti-mutant protein antibody should be an important tool for *in vitro* studies on the mutant receptor, including its use in immunoaffinity chromatographic purification of the mutant receptor and in growth control experiments. The antibody may also be labeled in order to follow the metabolic processing of the mutant receptor. The immunofluorescence studies described here suggest that antibody–mutant receptor complexes may follow the same metabolic pathway as EGF–EGFR complexes, with surface binding rapidly progressing to internalization. Second, *in vivo* growth control experiments may also be performed in nude animals bearing human glioma xenografts (21, 22) with the anti-peptide antibody serving as vehicle to target an isotope, toxin, or drug to the mutant EGFRs. Third, such a reagent will be useful to determine the prevalence rate of this particular mutation in the EGFR genes of tumors. In a preliminary screen of frozen-section tissue of biopsies from 35 glioblastomas, we have detected the mutation in 6 cases. Three of these cases were biopsies of tumors known to express mutant EGFRs, including tumor D-270 MG-Bx (9). Confirmation of the existence of the mutant EGFR in the 3 additional cases will require molecular analyses. The prevalence of this deletion mutation in glioblastomas, as judged by this initial screen, thus appears low relative to the nearly uniform mutation of c-Ki-*ras*, for example, in human pancreatic carcinoma (23), but is still at a significant level (17%). Further experiments, including those with a larger number of cases, will be necessary to precisely establish the prevalence of this and other mutations in the glioblastoma EGFR gene. It may be of interest to screen other tumors, such as squamous cell carcinomas, which amplify and rearrange the EGFR gene for the presence of this deletion mutation with this anti-peptide antibody. Finally, such an anti-mutant oncoprotein antibody may have clinical utility in the immunoimaging and immunotherapy of patients with glioblastomas expressing the mutant EGFR. The well-known antigenic heterogeneity of glioblastomas may be overcome by the use of this anti-peptide antibody, as the antibody binds to virtually every cell in the tumors expressing the mutant EGFR. An advantage of the anti-peptide antibody described here over antibodies previously raised against mutant oncoproteins such as *ras* p21 is the cell-surface expression of this EGFR mutation-site epitope compared to the intracellular localization of *ras*. This mutant EGFR fusion junction may then be more accessible *in vivo* for antibody localization and targeting.

In summary, the results of this study indicate that the knowledge of the amino acid sequence at the fusion junction of an in-frame deletion mutation in a human tumor allows for the generation of a probe (an antibody) that will preferentially bind the mutant oncoprotein. Numerous anti-peptide antibodies have been generated against linear sequences of the intact EGFR (11, 24, 25); here we report the generation, purification, and characterization of the first anti-peptide antibody preferentially reactive with mutant EGFR. This approach represents the custom design of a site-specific reagent for the mutational event in an individual patient's tumor. Analogously, anti-idiotype antibodies are custom-designed reagents for reaction with each patient's unique idiotypic determinants on B cells; these antibodies have been used to treat patients with leukemia and lymphoma (26). In contrast to anti-idiotypic antibodies, though, the anti-fusion junction peptide antibody may react, not with just one patient's tumor, but rather with the tumors of all patients with this particular EGFR deletion mutation. Immunoimaging and immunotherapy have been performed with antibodies reactive against brain tumors, including antibodies reactive against the intact EGFR in gliomas (27, 28). However, the molecules thus far targeted in brain tumors, such as the intact EGFR, are also expressed by normal tissues, thereby limiting the amount of antibody delivered to tumor and potentially leading to normal tissue toxicity. Targeting of mutant proteins and oncoproteins in human tumors should improve the selectivity of antibody delivery, as normal tissues should not express the mutants. Anti-peptide antibodies directed against specific mutation sites, such as the anti-fusion junction peptide antibody described here, represent powerful tools for the study of mutant oncoproteins in human tumors and may potentially be of clinical utility in the management of malignant human tumors.

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